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(54) Title: IMPROVED EMBRYOGENESIS PROCESS FOR INITIATION AND MATURATION

(57) Abstract

A method of producing mature somatic embryos (especially of conifers) to provide unexpected yield advantages comprising the steps of: (1) placing explants of the immature embryos on and/or in an initiation medium (the medium itself being part of the invention) or on a nurse culture itself on or in the initiation medium, (2) allowing the initiation to take place, and (3) (whether after optional storage and maintenance or not) maturing the initiated embryos on an appropriate maturation medium, wherein: (a) the initation and maturation medium may be the same or different, and wherein (b) (i) at least the initiating medium contains ABA and at least one amino acid, or (b) (ii) at least the initiating medium contains at least one amino acid and no ABA, and (c) at least the maturation medium contains ABA and at least one amino acid.

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IMPROVED EMBRYOGENESIS PROCESS FOR INITIATION AND MATURATION

The present invention relates to an improved conifer or other woody species embryogenesis process for initiation and maturation.

The initiation and maturation of embryogenic tissue and somatic embryos respectively has hitherto been part of a stagewise process from which explant to embryogenic tissue through to germination of somatic embryos and growth of somatic seedlings in the field has involved the 8 stages referred to hereinafter.

1. Initiation of embryogenic tissue
2. Maintenance of embryogenic tissue
3. Development of embryogenic tissue
4. Maturation of somatic embryos
5. Starvation and storage of somatic embryos
   (see New Zealand Patent Specification No. 272210)
6. Germination of somatic embryos
7. Growth of somatic seedling in greenhouse
8. Growth of somatic seedlings in field

Protocols for somatic embryogenesis for conifers typically involve several stages from initiation of embryogenic tissue through to somatic embryo maturation and germination. Patents providing background as to the use of embryogenesis to create somatic embryos include WO95/14373, US 5036007, US 5,034,326, US 5,041,382, US 4,957,866, AU 37150/93, US 5,294,549, South Africa 93/4807, and USA (CIP) 08/219879 (unpublished).

For initiation of conifer embryogenic cell lines whole gametophytes containing immature fertilised embryos or dissected immature fertilised embryos are used as explants. Explants are placed on several different "initiation media" to initiate embryogenic tissue either with or without growth regulators. The average percentage initiation over an entire seasonal initiation for radiata pine using whole gametophytes as explants has varied from 5 - 10%. The best percentage initiation has varied from 6 - 34 % for the best developmental window of sampling the explants from each seedlot. An initiated cell line is defined as an established and maintained cell line.

For maturation of conifer somatic embryos, initiated embryogenic cell lines are placed onto several media to maintain and develop the embryogenic tissue and multiply the number of embryo initials. Embryogenic tissue is then generally placed on a "maturation medium" to encourage the tissue to form mature embryos. Typically this maturation media contains abscisic acid (ABA). The percentage of initiated cell lines that are able to continue growth and form mature somatic embryos is typically 1 - 10%. Higher percentages of up to 25% have been obtained when a selection of 20 - 25% of the maintained embryogenic cell lines are placed on maturation media, ie; not all cell lines initiated are subsequently suitable for placement on maturation media.

The percentage of cell lines that are currently initiated using previously patented or published protocols is too low for adequate clonal representation within a family or cross and genetic diversity is not maintained satisfactorily.

Furthermore, from the embryogenic cell lines initiated the representation of clones forming mature embryos within a family is further reduced.

It is desirable from the perspectives of clonal testing, genetic diversity, process efficiency and cost effectiveness to have at least 50% initiation of clonal embryogenic cell lines and at least 30% formation of mature somatic embryos from those initiated embryogenic cell lines.

The present invention can achieve or at least approach this.

The present invention relates to various procedures and related methods and includes an embryogenic initiation medium which will result in changes to at least stages 1, the prospect of a merging of steps 2 and 3 with each of stages 1 and 4 and additionally potentiates the outcome at maturation step 4 for that embryogenic tissue capable of generating somatic embryos, the present invention therefore providing an increased efficiency over the prior art procedures.

It is to this that the present invention is directed.

In a first aspect the present invention consists in a **method of initiating embryogenic tissue from a source of immature embryos of a conifer or other woody species**, said method comprising:

placing explants of the immature embryos on and/or in an initiation medium or on a nurse culture itself on or in the initiating medium, and

allowing sufficient time for initiation to take place,

wherein (i) the initiation medium contains ABA and/or at least one amino acid, or (ii) the initiation medium containing just amino acids.

As used herein "woody species" includes the groups of species Eucalyptus family, Proteaceae, Myrtaceae, Rosaeceae, Punicaceae, etc.

Preferably said conifer immature zygotic embryo explants for the invention include those of *Pinus radiata* (or Monterey pine), hybrids of *Pinus radiata* and genetically modified *Pinus radiata*. This procedure is also applicable to other conifer species, viz, loblolly pine, Douglas fir, spruce species etc. and, of course, hybrids or genetically modified versions thereof.

Reference to "on" and "in" in respect of the media at least contemplates the use of gelled and/or liquid media.

Preferably said explant is not the whole megagametophyte and preferably is the dissected fertilised embryos at the bullet stage and before the pre-cotyledonary stage, 500 - (if radiata pine) celled embryo head developmental stage and most probably different called embryo head counts for other species which have different sized and shaped embryos.

Preferably the initiation medium does not contain traditional/conventional plant growth regulators such as auxins and cytokinins (eg; 2, 4-D, IAA, NAA, IBA, BAP, 2-IP, Zeatin, TDZ, etc). Nor preferably is it a medium for initiation with no growth regulators as outlined in South Africa 93/4807. But it does contain ABA and/or one or more amino acids.

Preferably ABA (Abscisic Acid) is present.

Preferably where said initiating medium is also to be used as the maturation medium ABA is present.

In another preferred form Abscisic Acid (ABA) may be absent but at least one amino acid is present. Preferably said amino acid that is present is one or more of the amino acids Arginine, Asparagine, Glutamine, Citrulline, Ornithine, Lysine, Alanine and Proline.

Preferably Glutamine is present.

Preferably at least one of Asparagine and Arginine is also present.

Preferably Glutamine, Asparagine and Arginine are present.

Preferably the initiation medium contains both ABA and at least one of the aforementioned amino acids and optionally several or all of the aforementioned amino acids.

Preferably said initiation medium includes in addition to said ABA and/or said at least one amino acid and other nutrient sources such as, for example, a source of essential macro and micro elements, vitamins, carbohydrates, inositol etc.

Preferably the initiation medium includes inorganic ions in the following ranges in concentration of the more significant ions in a preferred medium

ION	CONCENTRATION RANGE (mmoles/1)
NO_3	4.27
NH_4	0.5-6.8
Ca	0-0.9
Fe	0-0.15
Na	0-7
Zn	0-0.135
Cu	0-0.05
Mg	0-3.24

Preferably said ion concentrations are

ION	CONCENTRATION RANGE (mmoles/1)
NO_3	about 17.8
NH_4	about 1.96
Ca	about 0.17
Fe	about 0.10
Na	about 3.85
Zn	about 0.09
Cu	about 9.61×10^{-3}
Mg	about 1.62

In another embodiment preferably also present in the medium are the following inorganic ions or the total presence of inorganic ions is as follows

ION	CONCENTRATION (mmoles/1)
NO_3	17.80
NH_4	1.96
TOTAL N	19.76
P	1.96
K	14.16
Ca	0.17
Mg	1.62
Cl	$3.42x10^{-1}$
Fe	0.10
S	1.83
Na	3.85
B	0.13
Mn	$1.62x10^{-2}$
Zn	0.09
Cu	$9.61x10^{-3}$
Mo	$8.27x10^{-4}$
Co	$8.41x10^{-4}$
I	$6.02x10^{-3}$

Preferably also included are 5g/1-50g/l (w/v) Sucrose (preferably about 30g/l).

Preferably it also includes 3 - 8 grams gellan gum per litre (preferably about 5 grams) or other gelling agent (eg; agar or other).

Preferably it also includes 5 mg/l - 50 mg/l (w/v) Abscisic acid (ABA) (preferably about 15 mg/l).

Preferably the amino acids are present in the following ranges

ION	CONCENTRATION RANGE (mg/l)
Arginine	500-2,000
Asparagine	1,000-4,000
Glutamine	1,000-10,000
Citrulline	0-50
Ornithine	0-50
Lysine	0-50
Alanine	0-50
Proline	0-50

Preferably arginine is about 700, preferably asparagine is about 2,100 and preferably glutamine is about 7,300.

In another aspect the present invention consists in **a method of initiation of embryogenic tissue from a source of immature conifer or other woody species, said method comprising:**

placing explants of the immature fertilised embryos (directly or indirectly eg; nurse culture) on and/or in an initiation medium, and

allowing sufficient time for the initiation to take place,

wherein the initiation medium contains ABA and amino acids, or

wherein the initiation medium contains amino acids and no ABA.

Preferably conifers are the source of the explants.

Preferably the sufficient time is of the order of about 4-6 weeks.

Preferably the environment is in a sterile tissue culture vessel in a controlled environment at 15-28°C.

In a further aspect the present invention consists in **a method of initiation of embryogenic tissue from a source of immature conifer or other woody species embryos, said method comprising:**

placing explants of the immature fertilised embryos (directly or indirectly eg; nurse cells) on and or in an initiation medium, and

allowing sufficient time for the initiation to take place,

wherein the initiation medium is;

		Final Rate per Litre (mg)
Potassium Nitrate	(KNO_3)	1431
Magnesium Sulphate	($MgSO_4.7H_2O$)	400
Sodium Nitrate	($NaNO_3$)	310
Ammonium Dihydrogen Phosphate	($NH_4H_2PO_4$)	225
Calcium Chloride	($CaCl_2.2H_2O$)	25
Zinc Sulphate	($ZnSO_4.7H_2O$)	25
Boric Acid	(H_3BO_3)	8.0
Manganese Sulphate	($MnSO_4.H_2O$)	2.72
Copper Sulphate	($CuSO_4.5H_2O$)	2.4
Potassium Iodide	(KI)	1.0
Cobalt Chloride	($CoCl_2.6H_2O$)	0.2
Molybdic Acid	($Na_2MoO_4.2H_2O$)	0.2
EDTA - Disodium Salt		40
Iron Sulphate $7H_2O$		30
Nicotinic Acid		5.0
Thiamine HCl		5.0
Pyridoxine		0.5
Inositol		1000
Arginine		700
Asparagine		2100
Glutamine		7300
Citrulline		3.95
Ornithine		3.80
Lysine		2.75
Alanine		2.0
Proline		1.75

Abscisic Acid	5 to 50
Sucrose	5,000 to 50,000
Gelling Agent (GELRITE™)	3,000 to 8,000

Preferably the sucrose is about 30,000, the gelling agent is GELRITE™ about 4,500, and the abscisic acid is about 15.

In this respect reader is referred to South African Patent Specification No. SA 93/4807 where media is used for maturation only. The present invention recognises the usefulness and advantages of a media (not necessarily including ABA) for initiation and also (when preferably including ABA) for maturation.

In a further aspect the invention is a method of producing mature somatic embryos comprising the steps

(1) placing explants of the immature embryos on and/or in an initiation medium or on a nurse culture itself on or in the initiation medium,

(2) allowing the initiation to take place,

(3) (whether after optional storage and maintenance or not) maturing the initiated embryos on an appropriate maturation medium,

and wherein

(a) the initiation and maturation medium may be the same or different,

and wherein

(b) (i) at least the initiating medium contains ABA and at least one amino acid, or

(b) (ii) at least the initiating medium contains at least one amino acid and no ABA.

Preferably said at least one amino acid is selected from the group Arginine, Asparagine, Glutamine, Citrulline, Ornithine, Lysine, Alanine and Proline.

Preferably the maturation medium contains ABA and at least one amino acid.

The invention also consists in embryos thus matured.

In still a further aspect the present invention consists in an initiation and/or maturation media for embryogenic tissue, said medium comprising in addition to a

presence of ABA and at least ne amin acid, inorganic ions in the following ranges in concentration of the more significant ions in a preferred medium.

ION	CONCENTRATION RANGE (mmoles/1)
NO_3	4-27
NH_4	0.5-6.8
Ca	0.01-0.9
Fe	0.025-0.15
Na	0.5-7
Zn	0.023-0.135
Cu	$6x10^{-4}$-$5x10^{-2}$
Mg	0.405-3.24

Preferably said ion concentrations are

ION	CONCENTRATION RANGE (mmoles/1)
NO_3	about 17.8
NH_4	about 1.96
Ca	about 0.17
Fe	about 0.10
Na	about 3.85
Zn	about 0.09
Cu	about $9.61x10^{-3}$
Mg	about 1.62

Preferably said ion concentrations are as follows

ION	CONCENTRATION (mmoles/1)
NO_3	17.80
NH_4	1.96
TOTAL N	19.76
P	1.96
K	14.16
Ca	0.17
Mg	1.62
Cl	$3.42x10^{-1}$
Fe	0.10
S	1.83
Na	3.85
B	0.13
Mn	$1.62x10^{-2}$
Zn	0.09
Cu	$9.61x10^{-3}$
Mo	$8.27x10^{-4}$
Co	$8.41x10^{-4}$
I	$6.02x10^{-3}$

Preferably 5g/1-50g/l)w/v) Sucrose is also present (preferably about 30g/l).

Preferably 3 - 8 grams gellan gum per litre (preferably about 4.5 grams) or other gelling agent is also present.

Preferably it also includes 5 mg/l - 50 mg/l (w/v) Abscisic acid (ABA) (preferably about 15 mg/l).

Preferably the amino acids are present in the following ranges

ION	CONCENTRATION RANGE (mg/l)
Arginine	500-2,000
Asparagine	1,000-4,000
Glutamine	1,000-10,000
Citrulline	0-50
Ornithine	0-50
Lysine	0-50
Alanine	0-50
Proline	0-50

Preferably arginine is about 700, preferably asparagine is about 2,100 and preferably glutamine is about 7,300.

In yet a further aspect the present invention consists in and/or an initiation and maturation media for embryogenic tissue, said medium comprising;

		Final Rate per Litre (mg)
Potassium Nitrate	(KNO_3)	1431
Magnesium Sulphate	($MgSO_4.7H_2O$)	400
Sodium Nitrate	($NaNO_3$)	310
Ammonium Dihydrogen Phosphate	($NH_4H_2PO_4$)	225
Calcium Chloride	($CaCl_2.2H_2O$)	25
Zinc Sulphate	($ZnSO_4.7H_2O$)	25
Boric Acid	(H_3BO_3)	8.0
Manganese Sulphate	($MnSO_4.H_2O$)	2.72
Copper Sulphate	($CuSO_4.5H_2O$)	2.4
Potassium Iodide	(KI)	1.0
Cobalt Chloride	($CoCl_2.6H_2O$)	0.2
Molybdic Acid	($Na_2MoO_4.2H_2O$)	0.2
EDTA - Disodium Salt		40
Iron Sulphate $7H_2O$		30

Nicotinic Acid	5.0
Thiamine HCl	5.0
Pyridoxine	0.5
Inositol	1000
Arginine	700
Asparagine	2100
Glutamine	7300
Citrulline	3.95
Ornithine	3.80
Lysine	2.75
Alanine	2.0
Proline	1.75
Abscisic Acid	5 to 50
Sucrose	5,000 to 50,000
Gelling Agent (GELRITE™)	3,000 to 8,000

Preferably the sucrose is about 30,000, the gelling agent is GELRITE™ about 4,500, and the abscisic acid is about 15.

In yet a further aspect the present invention consists in an initiation embryogenic medium that is also effective as a maturation medium for initiated and maintained embryogenic tissue resulting from the initiation.

In still a further aspect the present invention consists in a method of increasing the efficiency of maturation of somatic embryos which comprises initiating and maturing the embryogenic tissue on a (preferably substantially common) medium which either

(a) contains ABA and amino acids,

(c) is of a composition substantially as follows;

		Final Rate per Litre (mg)
Potassium Nitrate	(KNO_3)	1431
Magnesium Sulphate	($MgSO_4.7H_2O$)	400
Sodium Nitrate	($NaNO_3$)	310

Ammonium Dihydrogen Phosphate	($NH_4H_2PO_4$)	225
Calcium Chloride	($CaCl_2.2H_2O$)	25
Zinc Sulphate	($ZnSO_4.7H_2O$)	25
Boric Acid	(H_3BO_3)	8.0
Manganese Sulphate	($MnSO_4.H_2O$)	2.72
Copper Sulphate	($CuSO_4.5H_2O$)	2.4
Potassium Iodide	(KI)	1.0
Cobalt Chloride	($CoCl_2.6H_2O$)	0.2
Molybdic Acid	($Na_2MoO_4.2H_2O$)	0.2
EDTA - Disodium Salt		40
Iron Sulphate $7H_2O$		30
Nicotinic Acid		5.0
Thiamine HCl		5.0
Pyridoxine		0.5
Inositol		1000
Arginine		700
Asparagine		2100
Glutamine		7300
Citrulline		3.95
Ornithine		3.80
Lysine		2.75
Alanine		2.0
Proline		1.75
Abscisic Acid		5 to 50
Sucrose		20,000 to 40,000
Gelling Agent (GELRITE™)		2,500 to 8,000

Preferably the sucrose is about 30,000, the gelling agent is GELRITE™ and is about 4,500, and the abscisic acid is about 15.

Preferably the efficiency is a potentiated increase in the efficiency in that the use of the initiation medium as set out in (c) or containing ABA and amino acids or

just amino acids increases the percentage of cell lines produced at initiation of the embryogenic tissue. (Figures 1 - 5). The cell lines initiated by the invention that go on to produce mature somatic embryos is also increased.

The present invention encompasses in the preparation of somatic embryos (particularly of conifers) a merged yet still sequential initiation and maturation procedure using a common or substantially common medium.

The present invention also envisages a procedure of generating mature somatic embryos which comprises:

initiation of embryogenic tissue from a source of immature conifer or other woody species embryos by a procedure of the present invention previously defined, and

maturing at least some of the embryogenic tissue to mature somatic embryos on the same medium but removing some of the initiated embryogenic tissue from the medium for bulking up and/or maintenance on a different media or for long term storage by cryo preservation before proceeding with additional maturation for producing much larger numbers of mature somatic embryos on the same or similar medium.

In a further aspect the present invention consists in mature embryos yielded by a method in accordance with the present invention and/or the use of an initiation and/or maturing media as previously set forth.

In yet a further aspect the present invention consists in a harvested product of a conifer or other woody species where the seed and/or seedling has resulted from the use of a method of the present invention and/or an initiation and/or maturing media as previously set forth.

In still a further aspect the present invention consists in wood, wood chips or cellulosic fibre derived from such a harvested product.

Indeed in prior art procedures the stages are as set out below.

1. Initiation of embryogenic tissue
2. Maintenance of embryogenic tissue (optional cryopreservation)
3. Development of embryogenic tissue

4. Maturation of somatic embryos
5. Starvation and storage of somatic embryos (NZ Patent Appl. No. 272210)
6. Germination of somatic embryos
7. Growth of somatic seedling in greenhouse
8. Growth of somatic seedlings in field

With the adoption of the present invention, initiation and maturation using the same medium, steps 1 to 4 of such a prior art procedure merge into a combined initiation and maturation stage, the duration of which is approximately 8-12 weeks.

1. Initiation and growth of embryogenic tissue (with optional cryopreservation)

1a.

(optional)Maintenance (with [optional] Cryopreservation)optional Development

2. Maturation of somatic embryos
3. Starvation and storage of somatic embryos
(preferably as in New Zealand Patent Specification No. 272210)
4. Germination of somatic embryos
5. Growth of somatic seedling in greenhouse
6. Growth of somatic seedlings in field

Somatic embryos produced by this invention can be used in the performance of the invention of our New Zealand Patent Specification No. 272210.

The methods and media of the present invention will now be described with particular reference to *Pinus radiata* as New Zealand's predominant exotic conifer species.

For initiation, whole megagametophytes are not used because of the uncertainty of whether a seed is fertilised or not. If not fertilised, no useful embryogenic tissue is formed.

Dissected fertilised embryos at the bullet stage and before the pre-cotyledonary stage, about 500 - 1000 called embryo head developmental stage. Other stages of development may be appropriate for other woody species, depending on the size and morphology of fertilised embryos.

Dissected embryos can be placed directly onto the initiation medium or onto a nurse culture. Vigorous growth of embryogenic tissue results.

Initiation media in at least some preferred forms for *Pinus radiata* did not contain traditional/conventional plant growth regulators such as auxins and cytokinins (ie; 2, 4-D, IAA, NAA, IBA and BAP, 2-IP, Zeatin, TDZ etc). The media of the present invention does contain ABA and/or some or all amino acids.

Typically initiation obtained with this method and of those initiated typically mature. These results have been shown over a range of eight genetically different families of *Pinus radiata*.

The performance of the present invention will now be described with particular reference to the accompanying drawings against procedures also for *Pinus radiata* as disclosed in South African Patent No. 93/4807.

In the accompanying drawings

Figure 1 is a comparison of initiation efficiencies relative to the total seeds extracted between the new method (that of the present invention) and the old method (that of South African Patent No. 93/4807),

Figure 2 is a comparison of initiation efficiencies relative to the number of surviving explants again comparing the new method and the old method,

Figure 3 is a comparison of contamination methods obtained by each initiation method;

Figure 4 is a comparison of explants into culture as a measure of screening ability; and

Figure 5 shows initiation results for various media types.

Statistical analysis (standard error of the mean) was carried out for all data in Figures 1 to 4.

Efficiency of initiation for radiata pine was assessed by two methods, one comparing the efficiency of the two methods relative to total seeds that were extracted from the cones and the other relative to the number of explants that survived being placed into culture. The "new" method is that described in the present invention for the initiation stage only. The "old" method and medium is that described in South African Patent No. 93/4807. Initiation for the purposes of Figures 1 and 2 is classified

as embryogenic tissue which has grown well enough to be classified as an embryogenic cell-line. Comparison of the two methods was only possible with crosses A96, B96, C96, D96, E96, G96, H96 and J96 initiated in late 1995/early 1996.

The total number of seeds tested for the old method was 16,927 for the purpose of Figure 1 and for the new was 28,414. For the purpose of Figure 2 the total number of surviving explants was 7,737 for the old and 9,974 for the new methods.

Figure 1 shows the difference in initiation efficiency between the two methods relative to starting seed counts. This shows that the new method has, on average, a significantly high efficiency in forming embryogenic cell lines.

The overall percentage initiation with the new method was 9.48% compared with 1.61% for the old method. At the individual cross level, the best method was not always the new method, when calculated relative to starting seed counts, as only crosses C96, E96 and H96 were significantly higher in initiation efficiency. This is thought to be related to other factors inherent to the cone (eg. stage of development, fertilisation efficiency) and to genetic factors.

Figure 2, which is a comparison of initiation efficiency relative to the number of surviving explants, shows a similar trend with the old method still showing a lower initiation efficiency. The overall average percentage initiation with the new method was 15.99% compared with 5.99% for the old method.

Embryogenic tissue of *Pinus taeda*, *Pinus pinaster*, *Pinus torreyana* and *Agathis australis* has been successfully initiated using the same method and media outlined in respect to the present invention.

The medium of or used by the methods of the present invention is believed optimal for *Pinus radiata* species for both initiation and maturation but nevertheless has a utility in relation to conifers and woody species in general, at least in respect of initiation, although for some species maintenance in a healthy state may cause difficulty with a common initiation/maturation medium. The *Pinus radiata* species maintained, in the common (preferred) initiation/maturation media, a healthy state for significant periods and tissue of *Pinus pinaster* and *Agathis australis* could be proliferated and maintained in a healthy state for at least four months. Explants for the

other species were selected at a similar stage of development as radiata pine, for initiation whereby the size and morphology of embryos differed.

Contamination levels obtained from the two initiation methods (Figure 3) show highly significant differences depending on the initiation method used. This was an unexpected new benefit of the common (substantially common) initiation/maturation media. The old method shows a much higher level of contamination, on average 20 times that of the new method. The overall average percentage contamination with the old method was 30.32% compared with 1.41% for the new method. The extremely high contamination levels of crosses A96 and C96 were associated with the use of old initiation media.

Another way of interpreting the new and old method for initiation is the screening comparisons shown by reference to Figure 4.

If an initiation method were to allow explants to be screened for initiation efficiency, then the use of media would be reduced, resulting in a cost saving. Comparison of the number of seeds initially extracted from the cone to the number of explants placed into culture can give a measure of the difference in the ability of the two methods to allow screening. The difference between the new and old method (Figure 4) shows there is an added ability of the new method to allow screening of explants. On average the new method allows 25% more screening than the old method. The average percentage of explants that could be placed in culture, if a screening procedure was used, using the new method would be 60.23% whereas with the old method would be 35.54%.

The addition or deletion of key components to the new initiation medium in this invention and the effect of these on initiation was evaluated in order to determine which factors were essential. Four treatments were evaluated.

1. Preferred medium containing ABA and amino acids (control).
2. Preferred medium containing amino acids and no ABA.
3. Preferred medium containing ABA and no amino acids.
4. Preferred medium containing no ABA and no amino acids.

Figure 5 demonstrates that the preferred media are those containing both ABA and amino acids or just amino acids, wherein percentage initiation values were 40.2%

and 43.5% respectively. Media without amino acids or with no amino acids and no ABA had significantly lower percentage initiation at 17.0 % and 11.1% respectively. Each media was tested with 4 different crosses.

The best percentage initiation of embryogenic cell lines from explants from the top 10 cones for both "new" and "old" methods (out of a total of 552 cones) was 42.91% for the new method and 26.29% for the old method. The initiation efficiency was calculated relative to the number of surviving explants and was statistically analysed (t - test significance < 0.003).

Percentage of cell lines forming mature radiata pine somatic embryos using "old" and "new" methods of initiation

	No. of Initiated Cell Line	No. of Mature Embryo-Forming Cell Lines	Percentage Maturation Efficiency
Old*	3642	338	9.3
New**	366	114	31.1

*Pooled data from 2 years (1993/94 and 1994/5 initiations) using "old" method.

**Incomplete data due to experiments ongoing at time of patent completion. Data on maturation from "old" method in Figures 1 - 5 not available, data shown for "new method above is from 4 crosses.

While in our preferred form of the invention we prefer to use the one medium for both initiation and maturation, the essence of the invention as claimed in respect of initiation and/or maturation media and methods would still be used where a different appropriate nutrient formulation is used for maturation from that used for initiation. Indeed the present invention envisages the use of an initiation or maturation media as claimed or indeed any appropriate initiation or maturation media to which, for maturation purposes at least, ABA and/or amino acids have been added. This is true whether for radiata pine or other conifer or other woody species.

This procedure of the present invention has an advantage of ensuring greater efficiencies of both initiation and maturation of any initiated embryogenic tissue, the

clones being passed through the system to the maturation and beyond to starvation and storage and germination.

CLAIMS:

1. **A method of initiating embryogenic tissue from a source of immature embryos of a conifer or other woody species, said method comprising:**

placing explants of the immature embryos on and/or in an initiation medium or on a nurse callus itself on or in the initiating medium, and

allowing sufficient time for initiation to take place,

wherein the initiation medium contains

(i) at least one amino acid, or

(ii) both ABA and at least one amino acid.

2. A method of claim 1 wherein the source is a conifer.

3. A method of claim 1 or 2 wherein said explant is not the whole megagametophyte and is the dissected fertilised embryos at 2 to 500 or more - (if a conifer species) celled embryo head stage.

4. A method of claim 3 when dissected at about the bullet or 500-1000- celled embryo head stage.

5. A method of any one of the preceding claims wherein the initiating medium does not contain traditional/conventional plant growth regulators such as auxins and cytokinins (eg; 2, 4D, IAA, NAA, IBA, BAP, 2-IP, Zeatin, TDZ, etc) but it does contain ABA and/or one or more amino acids.

6. A method of any one of the preceding claims wherein ABA (Abscisic Acid) is present.

7. A method of any one of claims 1 to 5 wherein ABA is absent but at least one amino acid is present being one or more of said amino acid(s) Arginine, Asparagine, Glutamine, Citrulline, Ornithine, Lysine, Alanine and Proline.

8. A method of claim 6 or 7 wherein Glutamine is present.

9. A method of claim 6, 7 or 8 wherein at least one of Asparagine and Arginine is present.

10. A method of claim 6 or 7 wherein Glutamine, Asparagine and Arginine are present.

11. A method of any one of the preceding claims wherein said initiating medium includes in addition to said ABA and/or said at least one amino acid other nutrient

sources such as, for example, a source of essential macro and micro elements, vitamins, carbohydrates, inositol etc.

12. A method of any one of the preceding claims wherein the initiation medium includes inorganic ions in the following concentrations

ION	CONCENTRATION RANGE (mmoles/1)
NO_3	4-27
NH_4	0.5-6.8
Ca	0-0.9
Fe	0-0.15
Na	0-7
Zn	0-0.135
Cu	0-0.05
Mg	0-3.24

13. A method of claim 12 wherein said ion concentrations are

ION	CONCENTRATION RANGE (mmoles/1)
NO_3	about 17.8
NH_4	about 1.96
Ca	about 0.17
Fe	about 0.10
Na	about 3.85
Zn	about 0.09
Cu	about 9.61×10^{-3}
Mg	about 1.62

14. A method of any one of claims 1 to 11 wherein also present in the medium are the following inorganic ions or the total presence of inorganic ions is as follows

ION	CONCENTRATION (mmoles/1)
NO_3	17.80
NH_4	1.96
TOTAL N	19.76
P	1.96
K	14.16
Ca	0.17
Mg	1.62
Cl	$3.42x10^{-1}$
Fe	0.10
S	1.83
Na	3.85
B	0.13
Mn	$1.62x10^{-2}$
Zn	0.09
Cu	$9.61x10^{-3}$
Mo	$8.27x10^{-4}$
Co	$8.41x10^{-4}$
I	$6.02x10^{-3}$

15. A method of any one of the preceding claims wherein the media contains 5g/1-50g/l (w/v) Sucrose.

16. A method of any one of the preceding claims wherein the media contains 3 - 9 grams gellan gum or other gelling agent.

17. A method of any one of the preceding claims wherein the amino acids are present in the following ranges

ION	CONCENTRATION RANGE (mg/l)
Arginine	500-2,000
Asparagine	1,000-4,000
Glutamine	1,000-10,000
Citrulline	0-50
Ornithine	0-50
Lysine	0-50
Alanine	0-50
Proline	0-50

18. A method of any one of the preceding claims including allowing at least partial maturation wherein said initiation media is used as the maturation media.

19. **A method of initiation of embryogenic tissue from a source of immature conifer or other woody species**, said method comprising:

placing explants of the immature fertilised embryos (directly or indirectly eg; nurse callus) on and/or in an initiation medium, and

allowing sufficient time for the initiation to take place,

wherein the initiation medium contains

(i) amino acids, or

(ii) ABA and amino acids.

20. A method of claim 19 wherein conifers are the source of the explants.

21. A method of claim 19 or 20 wherein the initiation medium contains ABA.

22. A method of any one of claims 19 to 21 wherein the sufficient time is of the order of about 4 weeks.

23. A method of any one of claims 19 to 22 wherein the environment is in a sterile tissue culture vessel at 15-28°C.

24. A method of initiation of embryogenic tissue from a source of immature conifer or other woody species, said method comprising:

placing explants of the immature fertilised embryos (directly or indirectly eg; nurse cells) on and or in an initiation medium, and

allowing sufficient time for the initiation to take place,

wherein the initiation medium is;

		Final Rate per Litre (mg)
Potassium Nitrate	(KNO_3)	1431
Magnesium Sulphate	($MgSO_4.7H_2O$)	400
Sodium Nitrate	($NaNO_3$)	310
Ammonium Dihydrogen Phosphate	($NH_4H_2PO_4$)	225
Calcium Chloride	($CaCl_2.2H_2O$)	25
Zinc Sulphate	($ZnSO_4.7H_2O$)	25
Boric Acid	(H_3BO_3)	8.0
Manganese Sulphate	($MnSO_4.H_2O$)	2.72
Copper Sulphate	($CuSO_4.5H_2O$)	2.4
Potassium Iodide	(KI)	1.0
Cobalt Chloride	($CoCl_2.6H_2O$)	0.2
Molybdic Acid	($Na_2MoO_4.2H_2O$)	0.2
EDTA - Disodium Salt		40
Iron Sulphate $7H_2O$		30
Nicotinic Acid		5.0
Thiamine HCl		5.0
Pyridoxine		0.5
Inositol		1000
Arginine		700
Asparagine		2100
Glutamine		7300
Citrulline		3.95
Ornithine		3.80
Lysine		2.75

Alanine	2.0
Proline	1.75
Abscisic Acid	5 to 50
Sucrose	5,000 to 50,000
Gelling Agent (GELRITE™)	3,000 to 8,000

25. **A method of producing mature somatic embryos** comprising the steps

(1) placing explants of the immature embryos on and/or in an initiation medium or on a nurse callus itself on or in the initiation medium,

(2) allowing the initiation to take place,

(3) (whether after optional storage and maintenance or not) maturing the initiated embryos on an appropriate maturation medium,

and wherein

(a) the initiating and maturation medium may be the same or different,

and wherein

(b) (i) at least the initiating medium contains ABA and at least one amino acid, or

(b) (ii) the initiating medium contains no ABA but includes at least one amino acid.

26. A method of claim 25 wherein the source is a conifer.

27. A method of claim 26 wherein ABA is present.

28. A method of claim 25, 26 or 27 wherein said at least one amino acid is selected from the group Arginine, Asparagine, Glutamine, Citrulline, Ornithine, Lysine, Alanine and Proline.

29. A method of any one of claims 25 to 28 wherein the maturation medium contains ABA and at least one amino acid.

30. **Embryos matured** by a method or using a method of any one of the preceding claims.

31. **An initiation and/or maturation media** for embryogenic tissue, said medium comprising in addition to a presence of ABA and at least one amino acid, inorganic ions in the following concentrations

ION	CONCENTRATION RANGE (mmoles/1)
NO_3	4-27
NH_4	0.5-6.8
Ca	0.01-0.9
Fe	0.025-0.15
Na	0.5-7
Zn	0.023-0.135
Cu	$6x10^{-4}$-$5x10^{-2}$
Mg	0.405-3.24

32. A media of claim 31 wherein said ion concentrations are

ION	CONCENTRATION RANGE (mmoles/1)
NO_3	about 17.8
NH_4	about 1.96
Ca	about 0.17
Fe	about 0.10
Na	about 3.85
Zn	about 0.09
Cu	about $9.61x10^{-3}$
Mg	about 1.62

33. A media of claim 31 or 32 wherein said ion concentrations are as follows

ION	CONCENTRATION (mmoles/1)
NO_3	17.80
NH_4	1.96
TOTAL N	19.76
P	1.96
K	14.16
Ca	0.17
Mg	1.62
Cl	$3.42x10^{-1}$
Fe	0.10
S	1.83
Na	3.85
B	0.13
Mn	$1.62x10^{-2}$
Zn	0.09
Cu	$9.61x10^{-3}$
Mo	$8.27x10^{-4}$
Co	$8.41x10^{-4}$
I	$6.02x10^{-3}$

34. A media of any one of claims 31 ro 33 wherein 5g/1-50g/l)w/v) Sucrose is also present.

35. A media of any one of claims 31 to 34 wherein 3 - 9 grams gellan gum per litre is present.

36. A media of any one of the preceding claims wherein amino acids are present in the following ranges

ION	CONCENTRATION RANGE (mg/l)
Arginine	500-2,000
Asparagine	1,000-4,000
Glutamine	1,000-10,000
Citrulline	0-50
Ornithine	0-50
Lysine	0-50
Alanine	0-50
Proline	0-50

37. **An initiation and maturation media** for embryogenic tissue, said medium comprising;

		Final Rate per Litre (mg)
Potassium Nitrate	(KNO_3)	1431
Magnesium Sulphate	($MgSO_4.7H_2O$)	400
Sodium Nitrate	($NaNO_3$)	310
Ammonium Dihydrogen Phosphate	($NH_4H_2PO_4$)	225
Calcium Chloride	($CaCl_2.2H_2O$)	25
Zinc Sulphate	($ZnSO_4.7H_2O$)	25
Boric Acid	(H_3BO_3)	8.0
Manganese Sulphate	($MnSO_4.H_2O$)	2.72
Copper Sulphate	($CuSO_4.5H_2O$)	2.4
Potassium Iodide	(KI)	1.0
Cobalt Chloride	($CoCl_2.6H_2O$)	0.2
Molybdic Acid	($Na_2MoO_4.2H_2O$)	0.2
EDTA - Disodium Salt		40
Iron Sulphate $7H_2O$		30

Nicotinic Acid	5.0
Thiamine HCl	5.0
Pyridoxine	0.5
Inositol	1000
Arginine	700
Asparagine	2100
Glutamine	7300
Citrulline	3.95
Ornithine	3.80
Lysine	2.75
Alanine	2.0
Proline	1.75
Abscisic Acid	5 to 50
Sucrose	5,000 to 50,000
Gelling Agent (GELRITE™)	3,000 to 8,000

38. **An initiation embryogenic medium that is also effective as a maturation medium for initiated and maintained embryogenic tissue resulting from the initiation, said medium being as claimed in any one of claims 31 to 37.**

39. **A method of increasing the efficiency of maturation of somatic embryos** which comprises initiating and maturing the embryogenic tissue on a substantially common medium which either

(a) contains ABA and amino acids,

(b) is of a composition substantially as follows;

		Final Rate per Litre (mg)
Potassium Nitrate	(KNO_3)	1431
Magnesium Sulphate	($MgSO_4.7H_2O$)	400
Sodium Nitrate	($NaNO_3$)	310
Ammonium Dihydrogen Phosphate	($NH_4H_2PO_4$)	225
Calcium Chloride	($CaCl_2.2H_2O$)	25
Zinc Sulphate	($ZnSO_4.7H_2O$)	25

Boric Acid	(H_3BO_3)	8.0
Manganese Sulphate	($MnSO_4.H_2O$)	2.72
Copper Sulphate	($CuSO_4.5H_2O$)	2.4
Potassium Iodide	(KI)	1.0
Cobalt Chloride	($CoCl_2.6H_2O$)	0.2
Molybdic Acid	($Na_2MoO_4.2H_2O$)	0.2
EDTA - Disodium Salt		40
Iron Sulphate $7H_2O$		30
Nicotinic Acid		5.0
Thiamine HCl		5.0
Pyridoxine		0.5
Inositol		1000
Arginine		700
Asparagine		2100
Glutamine		7300
Citrulline		3.95
Ornithine		3.80
Lysine		2.75
Alanine		2.0
Proline		1.75
Abscisic Acid		5 to 50
Sucrose		5,000 to 50,000
Gelling Agent (GELRITE™)		3,000 to 8,000

40. A method of claim 40 wherein (a) or (c) is used.

41. A method of claim 40 or 41 wherein (c) is used and the sucrose is about 30,000 and the gelling agent is GELRITE™ about 4,500.

42. A method of any one of claims 39 to 41 wherein the efficiency is a potentiated increase in the percentage of clones producing mature embryos from embryogenic tissue, the embryogenic tissue having been initiated as in Claim 1 - 24.

43. A method of any one of claims 39 to 42 wherein the efficiency is as a result of reduced contamination owing to the use of the same media for initiation and maturation.

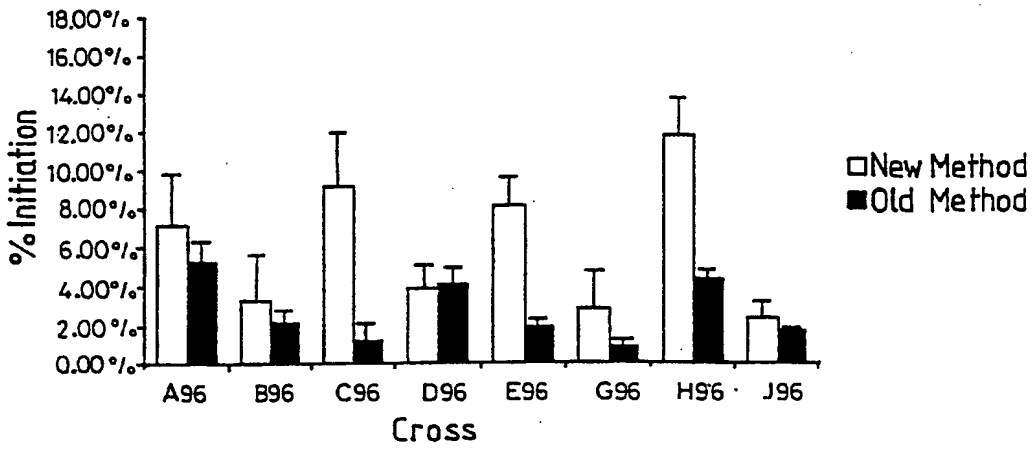
18.00%
16.00%
14.00%
12.00%
10.00%
8.00%
6.00%
4.00%
2.00%
0.00%
% Initiation
A96
B96
C96
D96
E96
G96
H96
J96
Cross
New Method
Old Method

FIG 1

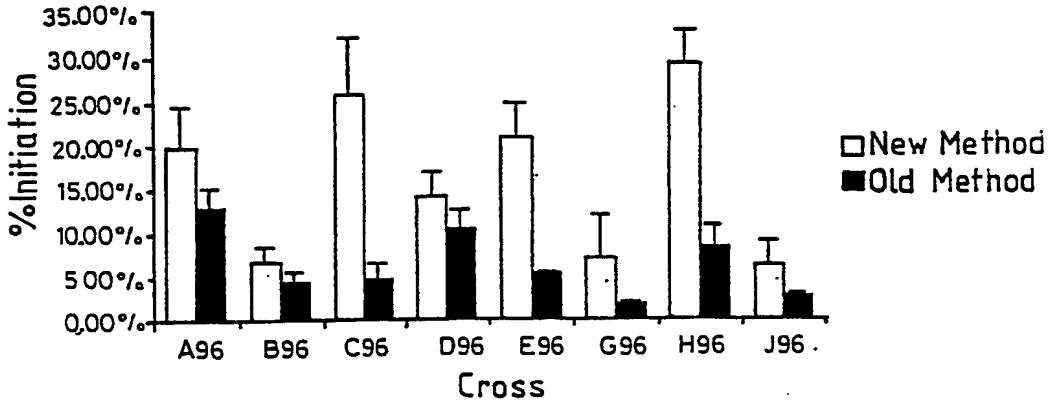
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
% Initiation
A96
B96
C96
D96
E96
G96
H96
J96
Cross
New Method
Old Method

FIG 2

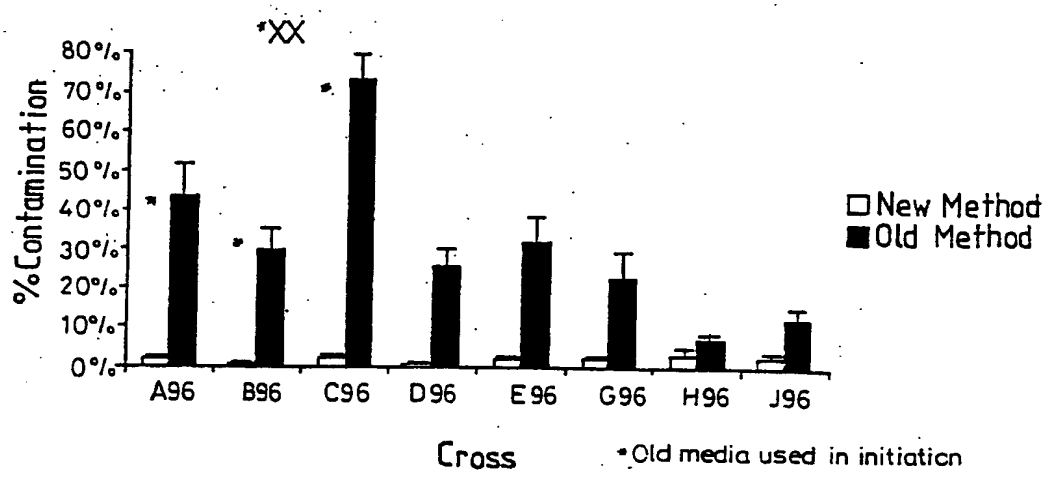
*XX
80%
70%
60%
50%
40%
30%
20%
10%
0%
% Contamination
A96
B96
C96
D96
E96
G96
H96
J96
Cross
New Method
Old Method
*Old media used in initiation

FIG 3

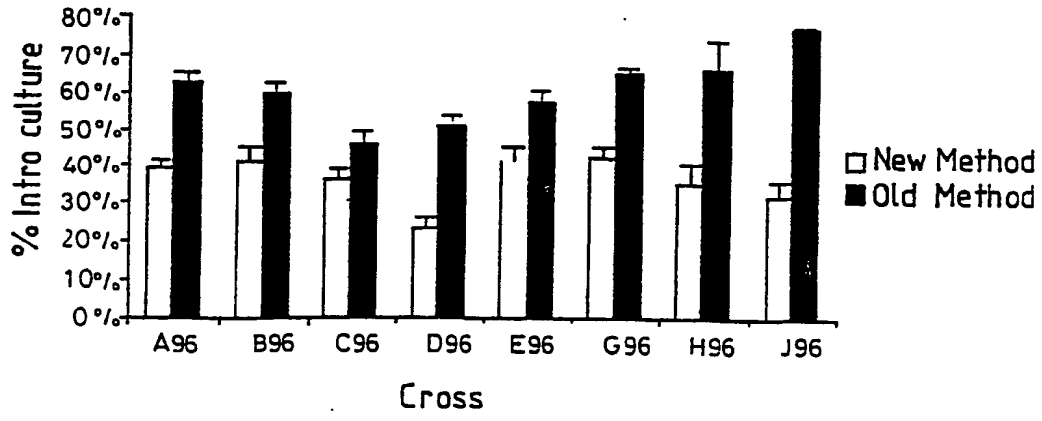
80%
70%
60%
50%
40%
30%
20%
10%
0%
% Intro culture
A96
B96
C96
D96
E96
G96
H96
J96
Cross
New Method
Old Method

FIG 4

Meda Code	ABA	Amino Acids	Cross No.	No. of Explants	% Initiation
34	✓	✓	D96	6	0
			E96	42	71.4
			G96	42	28,2
			J96	12	25,0
			mean	102	40.2
91		✓	D96	6	16.7
			E96	42	78.8
			G96	25	12.0
			J96	12	0
			mean	85	43.5
92	✓		D96	4	25 0
			E96	42	31 0
			G96	36	2 8
			J96	12	8 3
			mean	94	17.0
93			D96	0	
			E96	42	16 7
			G96	36	8 3
			J96	12	0
			mean	90	11.1

FIG 5

INTERNATIONAL SEARCH REPORT

'ernational Application No
PCT/NZ 96/00049

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01H4/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZA,A,934 807 (NEW ZEALAND FOREST RESEARCH INSTITUTE) 5 July 1993 cited in the application see the whole document ---	1-43
X	US,A,5 294 549 (WEYERHAUSER COMPANY) 15 March 1994 cited in the application see column 11, line 65 - column 15, line 63 see column 20, line 41 - column 21, line 15 --- -/--	1,2,6-9, 11, 19-21, 25-30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 September 1996	19. 09. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Herygers, J

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INTERNATIONAL SEARCH REPORT

ernational Application No
PCT/NZ 96/00049

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 19585 (CAMP CORPORATION) 14 October 1993 see page 10, line 8 - page 15, line 2 see page 23 ---	1,2,6-9, 19-21, 25-28,30
X	US,A,5 413 930 (BECWAR) 9 May 1995 see column 8, line 18 - column 22, line 12 ---	1,2,5-8, 16, 19-21, 25-30
X	WO,A,87 02701 (PLANT GENETICS INC.) 7 May 1987 see page 8, line 25 - page 12, line 15 -----	1,2, 7-11, 18-20, 25,26,30

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